Conjunctival bacterial and fungal flora in clinically normal sheep

Francesca Bonelli,1 Giovanni Barsotti,1 Anna Rita Attili,2 Linda Mugnaini,1 Vincenzo Cuteri,2 Silvia Prezioso,2 Michele Corazza,1 Giovanna Prezioso,1 Micaela Sgorbini1

ABSTRACT

Objectives: The aim was to identify conjunctival bacterial and fungal flora in clinically normal sheep.

Design: Prospective study.

Setting: Tuscany.

Participants: 100 eyes from 50 adult Massese female sheep were examined. The sheep included in the study were considered free of anterior ophthalmic abnormalities.

Primary and secondary outcome measures: Bacteria were identified by morphological assessment, Gram staining, biochemical tests. Identification of filamentous fungi was achieved at the genus level, and Aspergillus species were identified based on keys provided by other authors. Yeast colonies were highlighted, but not identified.

Results: Positive cultures were obtained from 100/100 eyes for bacteria, and from 86/100 eyes for fungi. A total of 14 types of bacteria and 5 types of fungi were isolated. Yeasts were isolated from 13/100 eyes. The most frequent fungal isolates were saprophytic fungi.

Conclusions: Conjunctival bacterial and fungal flora of clinically normal eyes were reported in sheep. The positivity obtained for conjunctival bacteria was higher compared to findings in the literature by other authors in the same species (100 per cent v 40 per cent), while our results were in line with a recent work performed on mouflons (Ovis Musimon) with a 100 per cent positivity for bacterial conjunctival fornix. In our survey, Gram-positive species were prevalent, as reported by other authors in different species. Few data are available in the literature regarding conjunctival fungal flora in healthy small ruminants. The prevalence of conjunctival fungal flora in this study was higher than findings reported in mouflons (86 per cent v 45 per cent). Differences in fungal prevalence may be due to different methods of managing herds, though further studies are required to verify this hypothesis. The similarities in bacterial and fungal isolates between sheep and mouflons suggest a genera pattern of flora, which could also be due to different methods of managing herds, though further studies are required to verify this hypothesis. Understanding the normal conjunctival flora is important in terms of possible implications in keratoconjunctivitis. Reports describing the normal conjunctival flora of sheep are scarce and old (Baker and others 1965, Spradlbrow 1968, Hopkins 1973, Baas and others 1977, Araghi-Sooreh and Hatami-Lorzini 2012). In clinically normal sheep, 60 per cent of eye swabs have been found to be negative for bacterial growth (Ramsey 1999). The most commonly isolated bacteria were similar to Branhamella (Neisseria) ovis and were recovered in small numbers. Other frequently isolated organisms were Micrococcus species and Streptococcus species. Less commonly isolated bacteria were included in the genera Corynebacterium, Achromobacter, Bacillus, Neisseria (other than N ovis), Staphylococcus, Pseudomonas, Moraxella and Escherichia, (Baker and others 1965, Spradlbrow 1968, Ramsey 1999, Waldridge and Colitz 2002). The fungi most frequently isolated from sheep and goats conjunctival fornix are Aspergillus species and Mucor species (Hopkins 1973, Baas and others 1977,
Waldridge and Colitz 2002). The aim of this work was to identify conjunctival bacterial and fungal flora in clinically normal sheep.

MATERIALS AND METHODS

A total of 100 eyes from 50 sheep were examined in August 2010. Approval to conduct this study was obtained from the Ethics Committee on Animal Experimentation of the University of Pisa (DL 116/92). All sheep were female, aged more than one year, and they all underwent similar management conditions. All the animals were considered healthy on the basis of clinical examination and free from anterior ophthalmic abnormalities, as determined by an ophthalmic examination performed after the conjunctival sampling.

The eye and periocular region were examined in normal light for gross abnormalities. Menace response, palpebral and corneal reflex tests were also performed. A Schirmer tear test was conducted on each eye using a commercial test strip (Dina strip Schirmer-Plus; GECIS sarl, Neung sur Beuvron, France). Tear production was recorded in millimetres wetting after 60 seconds. These procedures were performed in daylight or artificial light.

The adnexa and anterior segment of both eyes were examined with a binocular magnifying loupe, a transilluminator 3.5 V (Heine, Berlin, Germany), and a portable slit-lamp biomicroscope (SL-14, Kowa Company, Tokyo, Japan). Pupillary light reflexes were also evaluated in a dark stable.

Intraocular pressure was assessed by applanation tonometry (Tonopen-XL, Mentor, Norwell, Massachusetts, USA) after topical application of oxibuprocaine chlorhydrate 0.4 per cent (Benoxinato chlorhydrate INTES; ALFA INTES Industria Terapeutica Splendore S.r.l., Naples, Italy). Intraocular pressure was measured in each eye by the same operator. The total number of fungi and bacteria isolates were visually counted by the same operator. The number of colonies on each plate was converted to the number of bacteria per 1 ml of physiological solution (equal to the number of bacteria per eye) with the equation used in the literature (Ferguson and others 2003). Additionally, each conjunctival swab was inoculated in Tripticase Soya broth, incubated at 37°C for six hours and then streaked on whole media. The culture media used were: Columbia sheep blood agar, with and without Streptococcus Selective Supplement, Mannitol Salt agar, MacConkey agar (Oxoid, Milan, Italy). Plates were incubated at 37°C and examined for growth at 24, 48 and 72 hours. Representative colonies of bacteria were subcultivated onto Columbia blood agar plates and identified by morphological assessment, Gram staining, biochemical tests and, where necessary, using additional identification kits (Remel – Oxoid, Milan, Italy).

Mycological samples

Samples were plated onto Sabouraud dextrose agar (SDA, Oxoid, Milan, Italy) and malt extract agar (MEA, Oxoid, Milan, Italy), incubated at 25°C and examined daily from day 4 postincubation (p.i.), over a 21-day period to identify slow-growing organisms (Rosa and others 2003). Identification of colonies of filamentous fungi was achieved at the genus level on the basis of macroscopic and microscopic features of colonies, as described in the literature (Barnett and Hunter 1998). Aspergillus species were identified based on keys provided by other authors (Rapper and Fennell 1965). Yeast colonies were highlighted but not identified. Each test was carried out in duplicate to confirm the results. Colony forming units (CFU) were visually counted by the same operator. The total number of fungi and bacteria isolates per eye expressed in percentages (prevalence) in 100 normal eyes from 50 sheep was calculated.

RESULTS

Results regarding bacterial and fungal prevalence and CFUs are reported in Table 1. Positive cultures were obtained from 100/100 (100 per cent) eyes for bacteria and from 86/100 (86 per cent) eyes for fungi. A total of 14 species of bacteria were isolated: 10 types of Gram-positive bacteria and four Gram-negative bacteria. The Gram-positive bacteria were: five rods (Bacillus subtilis, Bacillus cereus, Bacillus thuringiensis, Bacillus licheniformis and Corynebacterium species) and five cocci (Enterococcus species, coagulase-negative staphyloccoci, Streptococcus γ-hemolytic, Staphylococcus aureus and containing 1 ml of physiological solution (Oxoid, Milan, Italy) and vortexed for 30 seconds. Subsequently, 100 μl aliquots were spread onto a Columbia agar plate containing 5 per cent sheep blood, which was incubated at 37°C for 24–48 hours. The same procedures were repeated for incubation in anaerobic conditions (Anagen Oxoid, Milan Italy). Colonies were counted by hand on both plates, using an illuminated colony counter when large numbers of colonies were present. The number of colonies on each plate was converted to number of bacteria per 1 ml of physiological solution (equal to the number of bacteria per eye) with the equation used in the literature (Ferguson and others 2003). Additionally, each conjunctival swab was inoculated in Tripticase Soya broth, incubated at 37°C for six hours and then streaked on whole media. The culture media used were: Columbia sheep blood agar, with and without Streptococcus Selective Supplement, Mannitol Salt agar, MacConkey agar (Oxoid, Milan, Italy). Plates were incubated at 37°C and examined for growth at 24, 48 and 72 hours. Representative colonies of bacteria were subcultivated onto Columbia blood agar plates and identified by morphological assessment, Gram staining, biochemical tests and, where necessary, using additional identification kits (Remel – Oxoid, Milan, Italy).

Bacteriological samples

On reaching the microbiology laboratory, the conjunctival swabs were placed aseptically into a sterile tube containing 1 ml of physiological solution (Oxoid, Milan, Italy) and vortexed for 30 seconds. Subsequently, 100 μl aliquots were spread onto a Columbia agar plate containing 5 per cent sheep blood, which was incubated at 37°C for 24–48 hours. The same procedures were repeated for incubation in anaerobic conditions (Anagen Oxoid, Milan Italy). Colonies were counted by hand on both plates, using an illuminated colony counter when large numbers of colonies were present. The number of colonies on each plate was converted to number of bacteria per 1 ml of physiological solution (equal to the number of bacteria per eye) with the equation used in the literature (Ferguson and others 2003). Additionally, each conjunctival swab was inoculated in Tripticase Soya broth, incubated at 37°C for six hours and then streaked on whole media. The culture media used were: Columbia sheep blood agar, with and without Streptococcus Selective Supplement, Mannitol Salt agar, MacConkey agar (Oxoid, Milan, Italy). Plates were incubated at 37°C and examined for growth at 24, 48 and 72 hours. Representative colonies of bacteria were subcultivated onto Columbia blood agar plates and identified by morphological assessment, Gram staining, biochemical tests and, where necessary, using additional identification kits (Remel – Oxoid, Milan, Italy).
The Gram-negative bacteria were: *Escherichia coli*, *Alcaligenes faecalis*, *Streptobacillus* species, and *Micrococcus* species. The most frequently isolated Gram-positive bacteria were *Bacillus subtilis* (48/100 eyes; 48 per cent), *Enterococcus* species, *Bacillus cereus* (33/100 eyes; 33 per cent) and *Bacillus thuringiensis* (15/100 eyes; 15 per cent). Of the Gram-negative bacteria isolated, we found 16/100 eyes (16 per cent) were positive for three fungi genera, 30/100 eyes (30 per cent) were positive for two fungi genera, and 49/100 (49 per cent) eyes were positive for one fungal species. Yeasts were isolated in 31/100 eyes (31 per cent), while 93/100 (93 per cent) eyes were positive for two or more bacteria. A total of five species of fungi were isolated: *Mucor* species, *Aspergillus* species, *Penicillium* species, *Alternaria* spp. and *Cladosporium* spp. Yeasts were isolated from 13/100 eyes (13 per cent). The most frequently isolated fungi were: *Mucor* species in 49/100 eyes (49 per cent), *Aspergillus* species in 31/100 eyes (31 per cent), and *Penicillium* species in 26/100 eyes (26 per cent). *Aspergillus* were identified as *A. niger* and *A. flavus* species. A total of 40/100 eyes (40 per cent) were positive for one fungus genus, 30/100 eyes (30 per cent) were positive for two fungi genera, and 16/100 eyes (16 per cent) were positive for three fungi genera; 4/100 (4 per cent) eyes were positive only for yeasts. CFUs ranged between 1 and 100.

All the bacteria and fungi isolated are reported in Table 1, along with the frequency of isolation per eye, and the mean CFU/ml recorded per microorganism for bacteria and CFUs per eye for fungi.

**CONCLUSIONS**

We investigated conjunctival bacterial and fungal flora in clinically normal eyes in sheep. All the eyes from the examined animals were positive for at least one microorganism. Bacteria were isolated in all the eyes examined, while fungi were present in 86/100 eyes. The positivity obtained for conjunctival bacteria was higher compared to findings in the literature by other authors in the same species (100 per cent v 40 per cent) (Spradbro 1968). On the other hand, our results were in line with a recent work performed on mouflons (*Ovis Musimon*) with 100 per cent positivity for bacterial conjunctival flora. In our survey, Gram-positive species were prevalent, as reported for other species (White and others 1983, Dubay and others 2000, Silvanose and others 2001, Pinard and others 2002, Andrew and others 2003, Cousquer and others 2010, Taddei and others 2010). The most frequent Gram-positive bacteria isolated (*Bacillus* species, *Enterococcus* species, *Corynebacterium* species, *Staphylococcus* species) were similar to bacteria isolated previously from mouflons (Petruzzi and others 2002). Regarding Gram-negative bacteria, the prevalence of *E. coli* and *A. faecalis* isolated in sheep was higher compared to other ruminants or herbivores (Gionfriddo and others 1992, Tuntivanich and others 2002, Andrew and others 2003, Taddei and others 2010, Johns and others 2011), while in mouflons, these bacteria were not isolated (Petruzzi and others 2002).

Fungi are considered part of the normal conjunctival mycoflora in many species (Samuelson and others 1984, Gionfriddo and others 1992, Tuntivanich and others 2002, Andrew and others 2003, Barsotti and others 2006, Nardoni and others 2007, Sgorbini and others 2008, 2010). Few data are available in the literature regarding conjunctival fungal flora in healthy small ruminants (Petruzzi and others 2002, Sgorbini and others 2010). The prevalence of conjunctival fungal flora in this study was higher than findings reported in mouflons (86 per cent v 45 per cent) (Petruzzi and others 2002). The most frequent fungal isolates in this study were saprophytic fungi, such as *Aspergillus* species, *Penicillium* species and *Mucoraceae*. These filamentous fungi are also the most frequent isolates in all the species examined by other authors, (Samuelson and others 1984, Gionfriddo and others 1992, Petruzzi and others 2002, Tuntivanich and others 2002, Andrew and others 2003, Barsotti and others 2006, Nardoni and others 2007, Sgorbini and others 2008, 2010, Johns and others 2011) but with a different prevalence. The prevalence of *Mucoraceae* found in this study was higher in sheep than has been found in the literature in other ruminants (Sgorbini and others 2010), while *Penicillium* species was lower than in mouflons.

### TABLE 1: Bacteria and fungi isolated from 100 eyes of 50 Massese sheep, their prevalence and CFU

<table>
<thead>
<tr>
<th>Gram-positive bacteria</th>
<th>Prevalence (%) (n=100)</th>
<th>CFU/ml (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>48</td>
<td>1×10⁵</td>
</tr>
<tr>
<td><em>Enterococcus</em> species</td>
<td>35</td>
<td>9×10⁴</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>33</td>
<td>1×10⁵</td>
</tr>
<tr>
<td><em>Corynebacterium</em> spp.</td>
<td>15</td>
<td>5×10⁴</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>10</td>
<td>11×10⁴</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>10</td>
<td>15×10⁴</td>
</tr>
<tr>
<td>Coagulase-negative</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>staphylococci</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td>8</td>
<td>8×10⁵</td>
</tr>
<tr>
<td>γ-hemolytic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6</td>
<td>12×10⁴</td>
</tr>
<tr>
<td>Micrococcus spp.</td>
<td>2</td>
<td>11×10⁴</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>28</td>
<td>7×10⁴</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em></td>
<td>16</td>
<td>1×10⁵</td>
</tr>
<tr>
<td><em>Streptobacillus</em> spp.</td>
<td>7</td>
<td>7×10⁴</td>
</tr>
<tr>
<td><em>Moraxella</em> spp.</td>
<td>3</td>
<td>6×10⁴</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mucor</em> spp.</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td><em>Aspergillus</em> spp.</td>
<td>31</td>
<td>67</td>
</tr>
<tr>
<td><em>Penicillium</em> spp.</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td><em>Alternaria</em> spp.</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td><em>Cladosporium</em> spp.</td>
<td>6</td>
<td>61</td>
</tr>
</tbody>
</table>

**CFU**, Colony forming unit
(Petruzzi and others 2002) and cows (Sgorbini and others 2010). The prevalence of *Aspergillus* species in sheep was higher compared to cows for some authors (Samuelson and others 1984), but similar compared to results reported in a recent study (Sgorbini and others 2010). Differences in fungal prevalence may be due to different methods of managing herds, though further studies are required to verify this hypothesis. The similarities in bacterial and fungal isolates between sheep and mouflons suggest a genera pattern of conjunctival colonisation by bacteria and fungi.

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REFERENCES


Hopkins J. B. (1973) Conjunctivitis associated with chlamydial polyarthritis in lambs. *Journal of the American Veterinary Medical Association* 163, 1157


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